

MASS PRODUCTION OF *Rhizopus oligosporus* SPORES AND THEIR APPLICATION IN TEMPEH FERMENTATION

INTRODUCTION

TEMPEH is a popular Indonesian food made by fermenting soybeans with *Rhizopus*. In 1967, we (Hesseltine et al.) reported that tempeh-like products also could be made from wheat, rice, other cereal grains and various combinations of them. Solid, cake-like tempeh has a mild and pleasant flavor when fried in vegetable oil and, therefore, has the potential for use in a variety of high-protein snacks.

Tempeh fermentation is characterized by its simplicity and rapidity. The lack of a suitable inoculum, however, could be a hindrance since it is essential that the inoculum be pure and that spores have a high degree of immediate germinability. Without these conditions, the fermentation process is almost inoperable. Traditionally, small pieces of tempeh from a previous fermentation serve as inoculum. The fungus is then propagated mainly by means of fast-growing mycelia. This practice can lead to contamination by undesirable microorganisms, and the inability of mycelia to survive adverse temperatures and dehydration makes mycelia unsuitable for long-term preservation of their viability. In this country both Hesseltine et al. (1963) and Steinkraus et al. (1960) developed pure culture fermentation of tempeh. However, preparing agar media for mass production of spores is expensive and time-consuming. Steinkraus et al. (1965) used freeze-dried, 4-day fermented soybeans as inoculum. Because we experienced failure and uncertainties with a similar preparation, we undertook to develop a tempeh inoculum having a high, viable spore count that would maintain its viability for a long time with minimal attention.

METHODS & MATERIALS

Cultures

Rhizopus oligosporus NRRL 2710 was maintained on slants of potato-dextrose-agar (PDA) at 4°C. Before each experiment, the organism was transferred to another PDA slant and incubated at 28°C for 7 days. A spore suspension for inoculation was prepared by adding 3 ml of sterilized, distilled water to each slant and shaking the culture vigorously for 1 min.

Spore production

Solid state fermentations, consisting of such grains as pearly wheat, cracked soybeans, polished rice and wheat bran, were used to prepare spores. In each 300-ml Erlenmeyer flask,

10g of a specific grain and various amounts of water were mixed and allowed to stand at room temperature for 1 hr with frequent shaking. The cotton-plugged flasks were autoclaved at 120°C for 20 min and then cooled to room temperature. Each flask was inoculated with 0.1 ml of spore suspension (10^5 viable counts). An incubation temperature of 32°C was used because preliminary work indicated that the sporulation of *R. oligosporus* was less at 25°C than at 32°C.

Viable spore count

The viable spores and other propagules of the fermentation mass, before and after freeze drying, were estimated by plate count. 1g of thoroughly mixed fermentation mass was aseptically weighed and transferred to a sterilized Waring Blendor containing 99 ml of sterilized water. After blending 2 min at high speed, serial dilutions were made from this initial dilution (1:100). A 1-ml suspension from each dilution was mixed with 10 ml of plate count agar of 45°C (0.5% bactopectone, 0.25% yeast extract, 0.1% dextrose and 1.5% agar) in a petri dish. After the dishes were held at 32°C for 20–24 hr, the colonies were counted.

Aerobic bacterial count

Aerobic bacterial counts were made by using plate count agar dishes containing 100 ppm Actidione®. Solutions of the antibiotic (10 mg/ml) were sterilized through Millipore® filters (0.45 μ) and added to agar at 45°C. The dishes were held at 32°C for 3 days, and colonies were counted daily.

Tempeh fermentation

Cracked soybeans or pearly wheat were

washed and soaked in water at room temperature for 30 min and boiled in excess water for 25 and 12 min, respectively, as described by Hesseltine et al. (1967). Freeze-dried spore preparations were mixed thoroughly with drain-dried, boiled grain. The inoculated grain was packed in petri dishes, trays or plastic tubing (5 × 15 cm) (Martinelli and Hesseltine, 1964) for incubation at 32°C for 18–22 hr.

Residue of water-extracted ground soybeans

Cracked soybeans were soaked in water at room temperature for 1 hr and washed free of hulls. The beans were wet ground in a Waring Blendor with additional water at a ratio of approximately 1:10 (w/v). The slurry was boiled for 15 min and filtered through a double-layered cheesecloth. The filtrate is usually referred to as soybean milk, from which tofu is made. The residue retained by the cheesecloth is the insoluble fraction.

RESULTS & DISCUSSION

Moisture requirement for sporulation

The moisture content of any substrate is of utmost importance in solid fermentation. When *R. oligosporus* was grown on various substrates for 4 days at 32°C, its growth and sporulation varied with the ratio of substrate to water. The viable spore counts of freeze-dried preparations made by growing *R. oligosporus* on four substrates—rice, pearly wheat, wheat bran and cracked soybeans—at three dif-

Table 1—Spore production of *Rhizopus oligosporus* grown on various substrates of three moisture levels

Substrate	Substrate:H ₂ O (w/v)	Substrate moisture (%) ^a	Viable spore count/g ^b
Rice	10:4	31	6.82
	10:6	40	8.03
	10:8	47	7.86
Wheat	10:4	34	6.70
	10:6	43	5.30
	10:8	50	5.30
Wheat bran	10:4	32	5.70
	10:6	40	6.95
	10:8	49	7.00
Soybeans	10:4	33	5.00
	10:6	39	5.70
	10:8	48	6.48

^a Substrate moisture is determined by drying at 110°C for 24 hr.

^b Counts are expressed as the logarithm of the numerical counts per g of freeze-dried preparation.

Table 2—Viable spore counts of *Rhizopus oligosporus* as affected by fermentation time, substrate and freeze drying

Day	Viable spore count/g ^a							
	Rice		Rice:wheat bran		Wheat		Wheat:wheat bran	
	B ^b	A ^c	B	A	B	A	B	A
4	9.05	8.01	9.21	7.82	8.01	6.91	8.94	8.25
5	9.08	7.85	9.33	7.86	9.08	7.16	9.11	7.68
6	8.48	7.21	8.64	7.10	8.24	6.97	8.64	7.15
7	8.19	7.52	8.54	7.59	7.93	7.31	8.19	7.45

Least significant difference = 0.49.

^a Counts are expressed as the logarithm of the geometric mean counts per g of sample on dry basis.^b B = before freeze drying^c A = after freeze dryingTable 3—Effect of storage time and temperature on the viability of freeze-dried *Rhizopus oligosporus* spores prepared from three substrates

Time (month)	Temp (°C)	Viable spore count ^a		
		Rice	Rice:wheat bran	Wheat:wheat bran
0		7.32	7.56	7.00
2	4	7.18	7.58	7.08
	22	6.00	6.85	5.90
4	4	7.40	7.46	7.23
	22	6.85	7.00	5.90
6	4	7.36	7.49	7.04
	22	6.60	7.08	5.95

^a Counts are expressed as the logarithm of the numerical counts per g of freeze-dried preparation.

ferent moisture levels for 4 days are recorded in Table 1. The fungus sporulated abundantly on rice, wheat bran and soybeans at a moisture level of 40% as well as 50%, but not at 30%. On the other hand, a 34% moisture level provided the best condition for sporulation of *R. oligosporus* on wheat; sporulation decreased as the moisture level increased.

Wheat bran, reported as a good substrate for spore production by *Aspergillus ochraceus* (Singh et al., 1968) and by *Hemispora stellata* (Sala and Burgos, 1972), also is a good substrate for sporulation of *R. oligosporus*. When the spore preparation made from wheat bran was used as inoculum for tempeh fermentation, the distinct dark-brown specks of wheat bran gave an unfavorable appearance to the final product. After extended fermentation of soybeans by *R. oligosporus*, an unpleasant odor often resulted, perhaps due to their high-protein content.

Therefore, soybeans and wheat bran alone were considered undesirable for making tempeh inoculum.

Interactions of fermentation time, substrate and freeze drying on viable spore counts

R. oligosporus was grown for 4–7 days at 32°C on four substrates: rice:water (10:6); rice:wheat bran:water (8:2:6); wheat:water (10:4); and wheat:wheat bran:water (8:2:6). On a dry basis, the viable spore counts per g preparation ranged from 6 to 257×10^7 before freeze drying and from 4 to 400×10^6 after freeze drying. Before freeze drying, the sample counts actually consisted of spores and other propagules, such as mycelial fragments. Therefore, low sample counts were expected after freeze drying. Not only do the mycelia succumb to adverse temperatures, but the percentage of spore germination is also affected adversely by freezing.

Data in Table 2 indicate the geometric mean spore counts from duplicate flasks as affected by fermentation time, substrate and freeze drying. As expected, sample counts taken before freeze drying were significantly higher than those taken after freeze drying. Analysis of variance indicated no significant interactions of freeze drying and substrates. Among the substrates tested for spore production by *R. oligosporus*, wheat was the poorest. The other three showed no differences.

Generally, adequate aeration is one of the environmental factors necessary for spore formation by *R. oligosporus*. The stickiness of the wheat substrate might have created an anaerobic condition unfavorable for sporulation. Attempts were made to improve the texture of wheat substrate by adding various amounts of wheat bran. As indicated in Table 2, a mixture of wheat and wheat bran (4:1) was a better substrate, on the one hand,

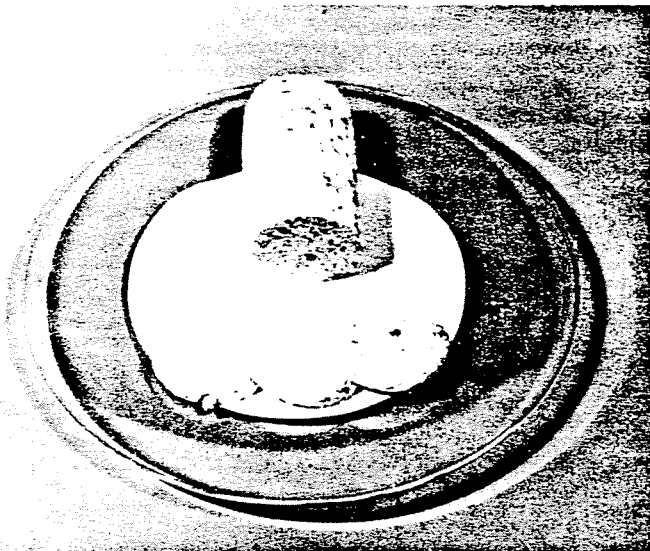


Fig. 1—Tempeh made from preinoculated soybeans after storing in a deep freezer for 2 months.

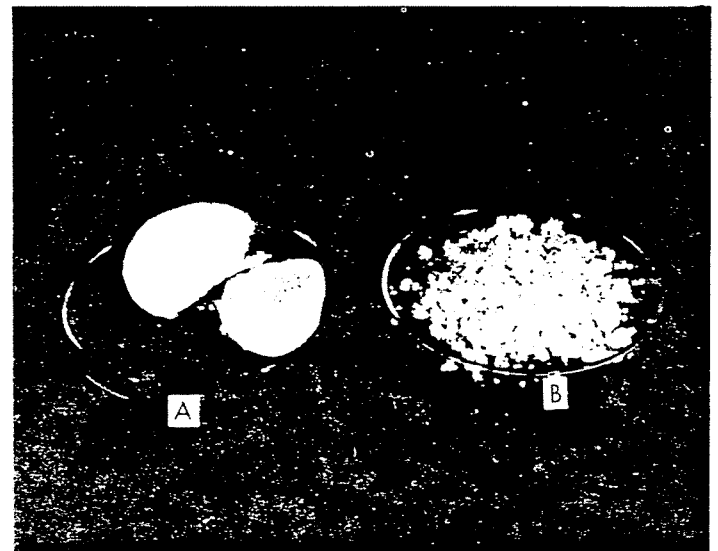


Fig. 2—Residue from water-extracted ground soybeans: (A) freshly prepared; (B) after drying at 100°C.

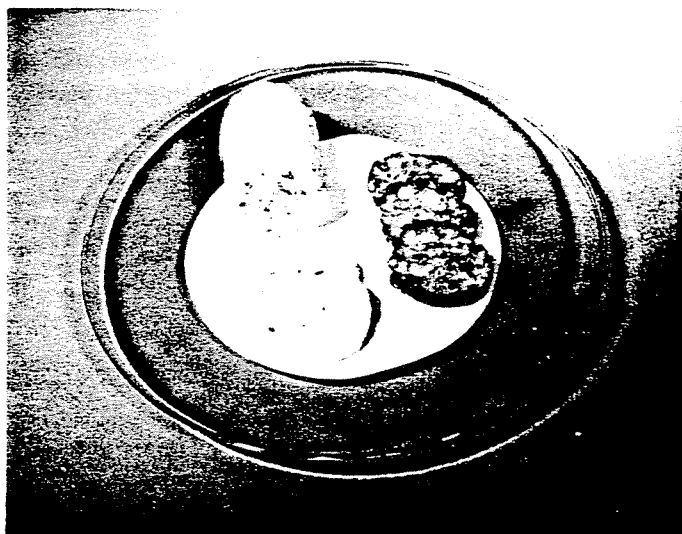


Fig. 3—Tempeh made from residue of water-extracted soybeans: left, fresh; right, cooked.

for spore formation by *R. oligosporus* than wheat alone. The addition of wheat bran to rice, on the other hand, had no effect on spore production.

Preliminary data indicated that spore production was much less after 3 days of incubation than after 4 days. Results in Table 2 showed adequate incubation time was from 4–5 days.

Based on these results, we suggest making *R. oligosporus* preparation by fermenting either rice, rice:wheat bran (4:1) or wheat:wheat bran (4:1) at a substrate to water ratio of 10:6 for 4–5 days at 32°C. The fermentation mass was then immediately freeze dried and ground into fine powder.

Storage stability

The freeze-dried and ground spore preparations were kept in closed plastic bags at 4°C or at room temperature (22°C) for 6 months. Their viable spore counts are summarized in Table 3. When the preparations were kept at 4°C for 6 months, the spore counts showed typical experimental variations and were comparable to their original counts; whereas, at room temperature, a significant decrease in viability of all three preparations was noted after 2 months. Thereafter, no further decrease was observed.

The possible contamination of the spore preparation by aerobic bacteria was checked. Bacterial contamination was not found to be a problem either during the process of fermentation or in storage.

Tempeh made with freeze-dried spores

To determine the amount of inoculum required to make satisfactory tempeh

from either soybeans or wheat, various amounts of spore preparations having 10×10^6 viable counts per g were added to 100g of cooked grain. The fermentation was carried out in petri dishes. When the level of inoculum varied from 0.01–0.15g (1×10^5 to 1.5×10^6 spores), the time required to complete fermentation ranged from 22 hr to less than 17 hr. We suggest using 1×10^6 spores per 100g of cooked beans or wheat, because fermentation time becomes too critical if the amounts of inoculum are larger. On the other hand, too small an amount of inoculum provides a chance for contaminating bacteria to grow. At the inoculum level of 1×10^6 spores per 100g cooked soybeans, good tempeh was also made by tray and package fermentations as described by Martinelli and Hesseltine (1964).

Inoculated soybeans were packed in plastic tubing (5 x 15 cm) and stored in a freezer at -15°C to defer fermentation until the tempeh was needed. After 2 months of storage, the bags were perforated with holes having a diameter of about 0.6 mm and a distance of about 0.5 cm by a small needle to allow aeration for mold growth and were incubated at 32°C. Good tempeh (Fig. 1) was made after an incubation time (20–22 hr) no greater than that required by freshly inoculated beans. Thus, preinoculated beans can be packaged, stored in a freezer and sold to be taken home and allowed to ferment in a warm place.

Tempeh made from residue of water-extracted ground soybeans

The residue from making soybean milk

and tofu, two main food products derived from water extraction of soybeans, has been considered a waste. Therefore, attempts were made to develop a palatable product from this residue by *R. oligosporus* fermentation. On a dry basis, the residue of water-extracted soybeans contains 32% protein, as determined by Kjeldahl digestion, and 20% oil as determined by ether extraction. Its moisture content is usually greater than 90%, so that the texture of the residue is too mashy to provide good growth for *R. oligosporus*. When moisture is reduced to less than 80% by drying at 100°C, this fraction appeared crumbly (Fig. 2) and was suitable for fermentation. After the fraction was inoculated with *R. oligosporus* spores, fermentation (Fig. 3) was completed after 20 hr at 32°C. Tempeh made from the residue of water-extracted soybeans has a texture and flavor similar to French-fried potatoes after deep-frying.

Hackler et al. (1963) reported that the water-insoluble fraction contains the highest quality protein, as measured by rat growth and protein efficiency ratio, among several soybean fractions studied: full-fat soybean flour, water-extract of soybeans, acid-precipitated curd and whey protein. Therefore, tempeh made from the residue of water-extracted soybeans is nutritious, as well as tasty.

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